Construction and Validation of Tissue Microarrays of Ductal Carcinoma In Situ and Terminal Duct Lobular Units Associated With Invasive Breast Carcinoma

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Abstract: Construction of tissue microarrays (TMAs) to efficiently characterize large sets of noninvasive epithelial lesions in the breast by immunohistochemistry is an appealing investigative approach, but presents technical challenges. We report methodologic studies performed to optimize methods for building TMAs from noninvasive breast tissues collected in a large case-control study of breast cancer. Using a manual arraying technique with 2.0-mm diameter needles, we constructed TMAs from specimens obtained from 32 women with breast cancer containing the following targets: (1) 28 terminal duct lobular units (TDLUs); (2) 28 ductal carcinomas in situ, and (3) 23 invasive carcinomas. Using careful target selection, we achieved representation of $\sim$80% of noninvasive targets with sustained preservation through section 30 of the TMAs. Immunohistochemical staining of TDLU targets demonstrated positive staining for estrogen receptor (ER) in 30.8% of tubules and for progesterone receptor (PR) in 50.0%. To establish an efficient method to evaluate staining results in TDLUs, we created a categorical scoring system to approximate the percentage of tubules containing positive stained cells ($<$ 10%, 10% to 50%, $\geq$ 50%), and compared the results with those obtained by tubule counting. Comparison between the two methods demonstrated exact agreement for 70.8% of ER and 79.2% of PR stains without two-category discrepancies. ER/PR expression levels in multiple (up to 4) noninvasive targets of the same tissue type (TDLU or DCIS) from a single block showed good correlation. These data suggest that it is feasible to produce TMAs of noninvasive breast structures, albeit with careful selection of targets, and that immunostains of such cores may permit efficient immunohistochemical characterization of peritumoral tissues. Additional exploration of this approach is needed.

Key Words: tissue microarrays (TMAs), DCIS, TDLUs, breast cancer

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Tissue microarrays (TMAs) represent an efficient method for preparing large numbers of samples for immunohistochemical analysis and other assays. Specifically, TMAs may be used to assay targets from hundreds of patients on single slides, permitting well-controlled staining in single batches, and thereby minimizing labor, cost, and assay variability. Assays performed on TMAs of invasive tumors collected in large studies may permit the analysis of risk factors, prognostic factors, treatment responses, and other outcomes by marker expression.

Construction of TMAs from large volume targets such as invasive cancers is highly effective, typically providing adequate representation in 80% to 90% of tissue cores.[1–3] However, methods commonly used to prepare TMAs of large targets are ill-suited for the preparation of TMAs from smaller targets such as cancer precursors or normal structures. Small targets are more difficult to sample and are frequently intermingled with tissue that is not of interest. In addition, because structures that seem small on cut sections are also likely to be thin, it is unclear whether the number of useful sections that can be cut from such TMA blocks justifies the effort required to produce them. Preparation of TMAs from noninvasive structures may be particularly useful for studies of natural history, etiology, and other investigations.

Accordingly, we developed and evaluated a method for preparing TMAs of ductal carcinoma in situ (DCIS) and terminal duct lobular units (TDLUs) of the breast. Our study focused on 2 outcomes: (1) percentage of targets adequately represented in TMAs and (2) number of deeper sections providing adequate target representation. Our ultimate goal is to use TMAs to immunhistochemically characterize invasive cancers, DCIS lesions,

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and normal TDLUs using specimens obtained from participants in a large population-based case-control study conducted in Poland. Developing TMAs of invasive and noninvasive tissues in this epideiomologic study will provide a novel resource for comparing marker expression in samples of invasive cancers, DCIS lesions, and TDLUs obtained from the same patient and for relating these results to breast cancer risk factors, tumor characteristics, treatment responses, and clinical outcomes.

MATERIALS AND METHODS

Tissue Samples

Study materials for this pilot project were collected as part of a population-based case-control study conducted in Warsaw and Lodz, Poland, in 2000–2003. Detailed description of the study design is presented elsewhere.[4] All subjects provided written informed consent to participate, and the protocol was reviewed and approved by human subjects review committees in Poland and the National Cancer Institute.

After removing tissues required for clinical diagnosis, 1 sample of tumor and 2 samples of nontumor tissue were collected for research purposes. Tissue samples were fixed in 10% buffered formalin and paraffin embedded in the usual manner.

Tissue specimens used for this pilot study were obtained from women who were ineligible for epidemiologic analyses (eg, questionnaire data were unavailable). Hematoxylin and eosin (H&E) stained slides of 100 tumor blocks containing invasive breast cancer were reviewed and areas of invasive carcinoma, DCIS, and TDLUs were marked. Noninvasive targets were marked on glass slides by placing ink directly over the targets, providing an approximate representation of the actual size and shape of the lesion. The size and proximity of the targets to the edge of the tissue or to adipose tissue were recorded. Extremely small lesions ($\leq$1 mm) and lesions that were intermingled with adipose tissue or approached the edge of the tissue were not chosen. TDLUs showing luminal dilatation, ductal hyperplasia, or calcifications were classified as fibrocystic changes and therefore, were not targeted.

Construction of TMAs

Using the mapped H&E stained slides as a guide, a 2.0-mm-diameter punch was taken from the selected target area in each donor block and put in a recipient paraffin block using a heated stage TMA construction device produced by Nippon Automatic Control Company of Japan. This device has a heated stage to soften the paraffin to allow removal of large tissue cores, without cracking the tissue block. Tissue blocks were prewarmed to 37°C for 10 to 30 minutes in an incubator and then transferred to the heated stage of the NACC arrayer. Cores were carefully collected by overlaying the marked H&E slide to localize the target, and then manually removed from the coring device with a stylus and, using small forceps, placed into a plastic template. Once all of the cores were placed, liquid paraffin was poured around the template to form the array recipient block. The block was then removed from the mold with the embedded plastic core holder and sectioned as normal. Sections were captured by floating paraffin ribbons on a water bath in a manner similar to conventional paraffin sectioning.

Microscopic Assessment

One hundred 5-μm-thick sections were cut from each TMA block. Every 10th section was stained with H&E to verify the target representation at different depths within the array blocks. For purposes of assessing the percentage of targets with adequate representation, we required cores of invasive cancer and DCIS to show $\geq$10% of the target visualized in the whole sections. Adequate cores of TDLUs were required to contain at least 10 ductules. Missing cores and cores containing nontargeted epithelial tissue types were both considered as inadequate. For cores containing TDLU targets, we attempted to count the number of tubular cross-sections microscopically while using a scanned and printed image of the core to assist in data recording.

Immunohistochemical Staining Procedure

Sections 11 and 12 of the TMA blocks were immunohistochemically stained for estrogen receptor (ER) and progesterone receptor (PR). The TMA slides were deparaffinized with xylene and then transferred through graded alcohols. Endogenous peroxidase activity was blocked by a 30-minute incubation in a 2.5% hydrogen peroxide/methanol buffer. Antigen retrieval was performed by boiling the slides in a pressure cooker filled with a sodium citrate buffer (pH 6.0). After antigen retrieval, the slides were incubated with 0.3% bovine serum albumin/1X Tris-buffered saline (TBS) for 1 hour at room temperature to reduce nonspecific background staining, followed by a series of 2-minute rinses in 1X TBS, 1X TBS/0.01% Triton, 1X TBS. Primary antibody [ERα (clone ID5, 1:50 dilution for 1 hour, DAKO) or PR (clone 636, 1:50 dilution for 1 hour, DAKO)] was applied for 1 hour at room temperature. After a series of TBS rinses as described above, bound antibody was detected by using an antirabbit horseradish peroxidase-labeled polymer secondary antibody from the DAKO Envision + System (DAKO, Carpinteria, CA). The slides were rinsed in the TBS series, visualized with a 10-minute incubation of liquid 3,3′-diaminobenzidine in buffered substrate (DAKO, Carpinteria, CA) for 10 minutes. Finally, the slides were counterstained with hematoxylin, and mounted with Immunomount (Shandon, Pittsburgh, PA).

Nuclear staining for ER and PR were evaluated in sections of TMAs by tissue type: invasive carcinoma, DCIS, and TDLU. For invasive and DCIS targets, the percentage of cells stained (0% to 100%) and staining intensity (negative, weak, intermediate, strong) were subjectively evaluated microscopically. For TDLU targets, the number of total tubules and the number of

tubules containing ≥ 1 cells with nuclear staining were counted separately and the percentage of positively stained tubules was calculated as (number of TDLUs containing immunopositive cells/number of total TDLUs) $\times$ 100. We then converted raw percentages to 3 categories based on the same scale we used for invasive cancer and DCIS targets ($< 10\%$; 10% to 50%; $\geq 50\%$). Assessing TDLUs by counting the total number of tubules and the number of tubules containing immunoreactive cells is very labor intensive and therefore, not feasible in large-scale studies. Accordingly, we reexamined the TMAs microscopically to subjectively assign categorical scores as above ($< 10\%$; 10% to 50%; $\geq 50\%$) for percentage of tubules containing immunoreactive cells based on visual assessment without counting. To validate the subjective estimation strategy, scores were compared with those obtained by counting.

For individual donor blocks that contained multiple noninvasive tissue targets of the same type (TDLU or DCIS), we attempted to remove each target in a separate core. Then we independently evaluated the immunostaining of each target in its respective core without reference to other cores of the same type prepared from that donor block. When multiple cores of a noninvasive tissue type were removed from a block, we averaged the raw data for percentage of cells stained and converted that value into a categorical result for comparison of cases. Counts of immunopositive tubules expressed as a categorical variable for each core were compared for multiple cores of the same tissue type removed from a single block.

Statistical Analysis

The percentage target retention was calculated by dividing the number of successfully punched targets by total number of identified targets of each tissue type. Frequency tables were constructed for ER/PR expression (a) by tissue type for all subjects, and (b) in multiple targets of the same tissue type from the same individual. All analyses were performed using SAS (version 9.1, SAS Institute).

RESULTS

TMA Construction

We constructed 2 TMA blocks consisting of 79 cores (normal = 28, DCIS = 28, invasive = 23) identified from 32 breast cancer patients (Fig. 1A). The size of the noninvasive targets ranged from 1.5 to 5.0 mm in diameter (median = 3.0 mm). Among the 56 noninvasive targets, there were 6 in proximity to the edge of the tissue and 1 surrounded by fat. In superficial sections, the percentage of cores with adequate representation (≥ 10 TDLUs or $\geq 10\%$ representation for invasive and DCIS targets) of invasive carcinoma was 91% as compared with 79% for noninvasive targets (DCIS and TDLUs combined). Figure 1B shows an H&E-stained tissue core containing normal TDLUs. All TDLU targets that were missed were small (2 to 3 mm in diameter). In contrast, the size of DCIS targets was not related to success in arraying. However, 3 out of 4 DCIS targets near the edge of the tissue were missed. For invasive carcinomas, satisfactory representation declined modestly to 78% in the 100th section, whereas for noninvasive structures, adequate representation remained essentially unchanged

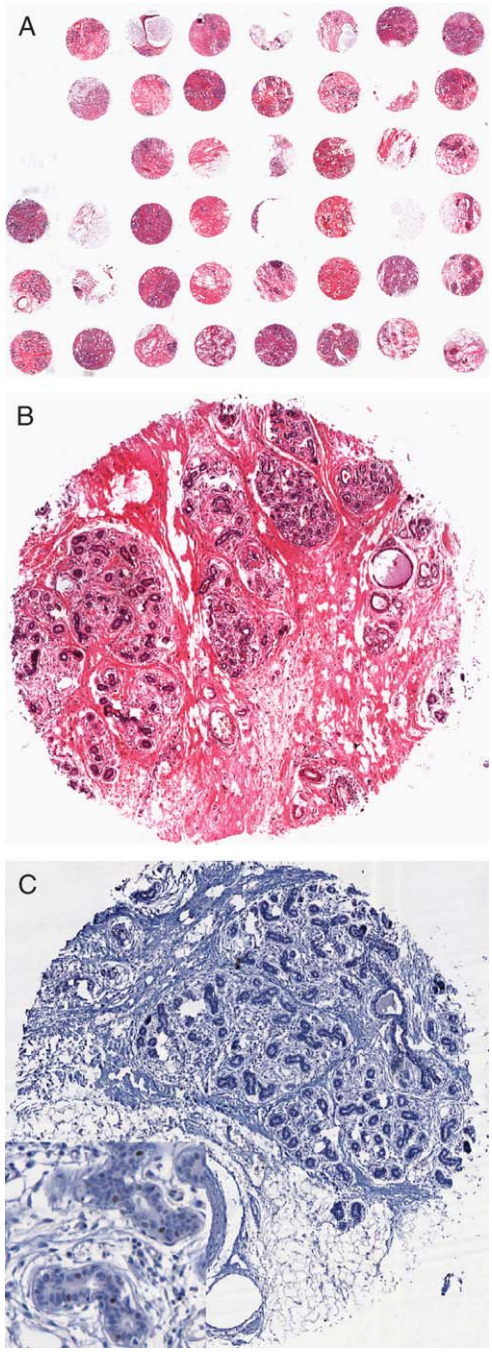

FIGURE 1. Images of a TMA of noninvasive breast structures. A, H&E-stained section of a TMA (low power). B, H&E-stained microarray tissue core demonstrating normal appearing TDLUs (high power). C, TMA core demonstrating strong nuclear staining for ER.

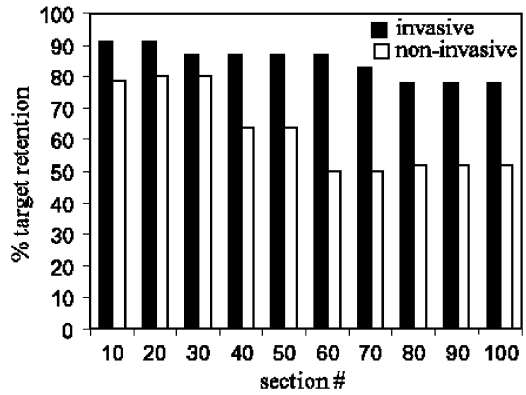

FIGURE 2. Successful target retention (%) for sections at different depth of the array blocks.

through section 30 and then, declined to 64% at section 40 and 52% at section 100 (Fig. 2).

Immunohistochemical Detection of ER and PR

In total, ER/PR staining results were obtained from 37 invasive targets (23 cases), 22 DCIS targets (13 cases), and 24 normal targets (13 cases) (Fig. 1C). The median percentage and range of ER expression in invasive carcinoma, DCIS, and TDLU targets were 45.0% (0% to 95.0%), 50.0% (0% to 90.0%), and 30.8% (0% to 81.8%), respectively. The corresponding numbers for PR were 20.0% (0% to 95.0%), 20.0% (0% to 100.0%), and 50.0% (1.9% to 81.3%).

Evaluation of ER/PR Expression in Normal TDLUs

The number of total tubules in TDLU targets ranged from 20 to 322 per core (median = 78). The percentage of ER/PR positive tubules varied greatly among different subjects with median results of 30.8% for ER and 50.0% for PR. Comparison of categorical results for the percentage of immunopositive tubules (< 10%; 10% to 50%; ≥ 50%) based on subjective estimation and counting demonstrated 70.8% concordance for ER and 79.2% for PR. All discordant measurements were within one category.

Comparison of ER/PR Staining in Multiple Targets of the Same Tissue Type From a Single Block

Numbers of targets containing a particular tissue type (TDLU or DCIS) from a single tumor block varied from 1 to 4. For 12 donor blocks which contained more than one successfully arrayed TDLU or DCIS targets (6 had multiple TDLU targets and 6 had multiple DCIS targets), we compared intrablock results for staining among these multiple cores to compare expression. There was good agreement in ER/PR staining (both percentage and intensity) among multiple targets of the same type from a single block for both TDLU and DCIS targets. All discordances were between adjacent categories (Table 1).

DISCUSSION

Although the use of TMA technology to immunohistochemically characterize large sets of noninvasive lesions represents an appealing investigative approach, efforts to achieve this goal have faced technical limitations. In this pilot study, we achieved successful arraying of ~80% of carefully selected noninvasive targets with good retention of representation of lesions through the 30th section of TMAs. Although our success rate for arraying noninvasive targets was less than that achieved for invasive lesions and the number of useful array sections available per TMA block was considerably fewer, we believe that construction of noninvasive arrays is technically feasible in large studies. Our data represent an improvement over previous reported data in which only 52% of DCIS targets could be adequately arrayed.[5] Compared to that study, we used larger needle size (2.0 mm vs. 0.6 mm) and carefully selected targets

TABLE 1. Comparison of ER/PR Staining Results of Multiple Cores Taken from a Single Specimen

	ER % Positivity/Intensity*				PR % Positivity/Intensity*			
	Core 1	Core 2	Core 3	Core 4	Core 1	Core 2	Core 3	Core 4
TDLU								
Case 1	≥ 50%/I	≥ 50%/I	≥ 50%/S	≥ 50%/I	≥ 50%/S	10% to 50%/I	10% to 50%/S	≥ 50%/I
Case 2	< 10%/W	< 10%/W			< 10%/W	< 10%/W		
Case 3	< 10%/W	10% to 50%/W	10% to 50%/I		≥ 50%/S	≥ 50%/S	≥ 50%/S	
Case 4	10% to 50%/W	10% to 50%/W	< 10%/W		10% to 50%/I	10% to 50%/I	10% to 50%/I	
Case 5	10% to 50%/W	10% to 50%/I			< 10%/W	< 10%/W		
Case 6	10% to 50%/W	≥ 50%/I	10% to 50%/I		10% to 50%/I	≥ 50%/I	10% to 50%/I	
DCIS								
Case 1	≥ 50%/I	≥ 50%/I	≥ 50%/W	10% to 50%/W	≥ 50%/S	≥ 50%/S	≥ 50%/S	≥ 50%/I
Case 2	10% to 50%/I	≥ 50%/I			10% to 50%/W	10% to 50%/I		
Case 3	≥ 50%/I	≥ 50%/I			≥ 50%/I	≥ 50%/S		
Case 4	10% to 50%/I	≥ 50%/S			< 10%/I	< 10%/W		
Case 5	< 10%/N	< 10%/N			10% to 50%/S	< 10%/I		
Case 6	≥ 50%/S	≥ 50%/S			≥ 50%/S	≥ 50%/S		

*Percentage positive: < 10%, 10% to 50%, ≥ 50%; Intensity: N indicates negative; W, weak; I, intermediate; S, strong.

(> 1.0 mm, not intermingled with adipose tissue or at the edge of tissue). In our experience, target selection and use of large core needles are critical for building noninvasive TMAs.

This pilot study represents the culmination of prior work in which we tried other methods for preparing noninvasive arrays (unpublished observations). Previous experimentation in which 0.6 mm in diameter core needles were used to remove tissue cores from donor blocks and place them into recipient blocks either in the usual orientation (perpendicular to the surface of the recipient block) or longitudinally (parallel to the surface of the recipient block) yielded unsuccessful results. Later, we achieved better success by using larger needles (2.0 mm) and arraying cores in the conventional orientation using the Beecher manual tissue arrayer MTA-1 (Sun Prairie, WI). We also determined that targets measuring less than 1.0 mm in greatest dimension, those that are intermingled with adipose tissue and structures that have their long axes oriented parallel to the plane of sectioning are difficult to effectively array. Even when small targets are successfully removed from donor blocks they sometimes cannot be effectively transferred into recipient blocks, resulting in loss of the structure in both the donor and recipient blocks. Use of large-sized cores substantially reduces the maximum number of cores that can be included in one array ($\sim$50 cores/array). Therefore, preparation of arrays of noninvasive targets is less efficient than arraying invasive cancers, but still provides substantial advantages over staining 1 section per case.

Previous studies of invasive breast cancers have shown that immunohistochemical characterization based on staining 1 or 2 cores provides generally accurate representation of the results obtained by staining an entire tissue section.[1,6,7] Given that noninvasive structures are often thin, and therefore, could be exhausted by sectioning the entire block before arraying, we chose to compare immunostain scores from several targets within a block rather than to compare results for arrays to whole sections. Although our results were limited to 12 blocks (6 containing multiple TDLU and 6 containing multiple DCIS targets) containing multiple noninvasive targets, this preliminary work suggests that a single core might provide a reasonable measure of expression for structures in the small region of the breast that comprises one block. A much larger study would be required to assess the range of heterogeneity demonstrated for each specific marker within larger areas or within an entire breast. If assessing noninvasive tissues near a tumor represents the relevant measure for testing a specific hypothesis, for example, the evaluation of peritumoral field changes, then large variation in marker expression within remote parts of the breast may be less of a concern. However, if measuring diffuse changes throughout the breast is an important objective and variation in expression varies widely by quadrant, then more extensive representation of tissues within TMAs will be required.

In general, multiple noninvasive targets of interest with ideal size and location are rarely available in breast specimens, even when additional sectioning is used. In a recent review of 1200 breast cancer blocks and 2400 matched adjacent normal tissue blocks collected from the Polish breast cancer case-control study, we identified $\sim$780 blocks that contain noninvasive targets (normal TDLUs and DCIS) deemed suitable for arraying based on the suitability criteria listed above. Less than 40% of these blocks had multiple targets of the same tissue type within a single block. Therefore, restricting array preparation to specimens containing multiple noninvasive targets would limit inclusion to a minority of potential cases, an approach which itself could lead to biased results.

Scoring normal TDLUs for marker expression is another challenge. With the assistance of color prints of computer-enlarged images of each core containing normal targets, we attempted to count the total number of tubules and positively stained tubules in each core. Though estimating tubule numbers by counting rather than subjective evaluation may provide more precise measurements, counting tubules is not practical in a large-scale study with thousands of cores to count. Based on the distribution of quantitative scores obtained from tubule counting, we created a subjective categorical scoring system for ER/PR staining results in TDLUs. Comparison of the 2 measurements showed good correlations suggesting that subjective scoring may be adequate. The development of a relatively inexpensive, automated system for reading such cores would be a great advance.

In summary, our data suggest that production of TMAs of noninvasive breast structures is feasible, although it requires careful selection of targets. Immunostains of such arrays may facilitate immunohistochemical characterization of peritumoral tissues. Further exploration of this technique is warranted. Since the completion of this pilot study, we have used a similar technique to construct TMAs of 1190 noninvasive targets with successful representation of $\sim$74%.

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